OPIC
OFFICE DE LA PROPRIÉTÉ INTELLECTUELLE DU CANADA

CIPO
CANADIAN INTELLECTUAL PROPERTY OFFICE

(12) (19) (CA) **Brevet-Patent**

(11) (21) (C) **2,101,942**
(86) 1992/02/07
(87) 1992/08/20
(45) 2001/01/30

(72) Dean, Richard T., US
(73) Diatide, Inc., US

(51) Int.Cl.[6] C07K 1/13, A61K 49/02
(30) 1991/02/08 (653,012) US

(54) **POLYPEPTIDES MARQUES AU TECHNETIUM-99m POUR L'IMAGERIE**

(54) **TECHNETIUM-99m LABELED POLYPEPTIDES FOR IMAGING**

(57) L'invention se rapporte à des techniques d'imagerie par radiomarquage pour la représentation du corps de mammifères. L'invention se rapporte en particulier à des réactifs marqués au technétium 99m pour de telles techniques d'imagerie. L'invention décrit des peptides qui lient le technétium 99m et qui peuvent être ciblés sur des sites spécifiques dans le corps d'un mammifère.

(57) The invention relates to radiolabeled imaging of a mammalian body. The invention in particular provides for reagents labeled with technetium-99m far such imaging. The invention provides peptides which bind technetium-99m and which can be targeted to specific sites within a mammalian body.

ABSTRACT

The invention relates to radiolabeled imaging of a mammalian body. The invention in particular provides for reagents labeled with technetium-99m for such imaging. The invention provides peptides which bind technetium-99m and which can be targeted to specific sites within a mammalian body.

TECHNETIUM-99m LABELED POLYPEPTIDES FOR IMAGING

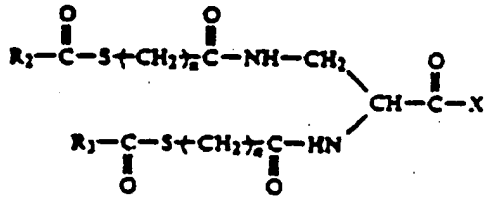
BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to radiodiagnostic reagents and, more particularly, to polypeptides useful for producing technetium (Tc-99m) labeled radiodiagnostic agents. The invention relates to Tc-99m labeled reagents, kits for making such reagents, and methods for using such reagents.

Description of the Prior Art

U.S. Patent No. 4,861,869 (Nicolotti) describes coupling agents of the formula:



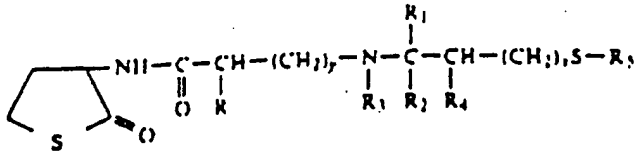
wherein R_2 and R_3 are the same or different and each represents a radical selected from the group consisting of alkyls having from 1 to 6 carbon atoms, aryls having from 6 to 8 carbon atoms and aklaryls having 7 to 9 carbon atoms, any of which can be substituted with one or more hydroxyl, alkoxy, carboxy or sulfonate groups; n is either 1 or 2; and X is an activating group capable of forming an amide bond with an alpha or beta amino group of a biologically useful protein or polypeptide molecule.

U.S. Patent No. 4,861,869 also describes compounds such as S-benzoylmercaptoacetylglycylglycylglycine.

The coupling agents are bound to large peptides such as antibodies or fragments thereof and complexed to Tc-99m.

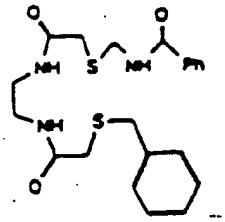
B

U.S. Patent Nos. 4,571,430, 4,575,556 and 4,434,151 (Byrne et al.) describe compounds of the formula:



wherein R is hydrogen or lower alkyl, R_1 and R_2 are individually hydrogen or lower alkyl or taken together form oxo; R_3 is an amino protecting group where R_1 and R_2 taken together form oxo; R_4 is hydrogen or lower alkyl; R_5 is hydrogen or a thiol protecting group; and y and z are integers from 0 to 2; which are bifunctional chelating agents and as such can couple radionuclides to terminal amino-containing compounds capable of localizing in an organ or tissue which is desired to be imaged.

Bryson et al., *Inorg. Chem.* 27: 2154-2161 (1988) and *Inorg. Chem.* 29: 2948-2951 (1990), describes thiolate ligands for complexing with technetium of the formula:



European publication no. EP188256 filed January 13, 1986, Fritzberg, Alan R. describes dithio, diamino, or diamidocarboylic acids or amine complexes useful for making technetium imaging agents.

Other references of interest include Khaw et al, *J. Nucl. Med.* 23: 1011 (1982); Rhodes, B.A. *Sem. Nucl. Med.* 4: 281 (1974); Davidson et al., *Inorg. Chem.* 20: 1629 (1981); and Byrne and Tolman, *J. Nucl. Med*

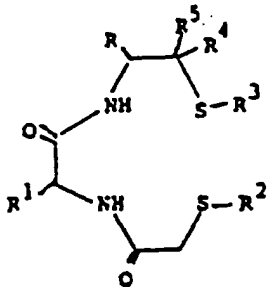
B

24: 126 (1983). *See* particularly Fritzberg et al., *J. Nucl. Med.* 23: 592 (1982); Fritzberg et al., *ibid.* 23: 17 (1982), for descriptions of mercaptoacetyl derivatives of ethylene diamine carboxylic acid derivates. *See also* U.S. Patent Nos. 4,434,151, 4,444,690 and 4,472,509.

European publication no. EP284071 filed March 24, 1988, Fritzberg, Alan R. et al., describes various S-protected mercaptoacetylglycylglycine chelating groups bound to large proteins such as antibodies.

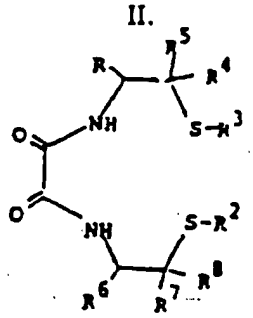
European publication no. EP135160 filed August 17, 1984, Davison, Alan et al., describes technetium complexes of compounds of the formula I and II:

I.



and

II.



wherein R and R_6 are each selected from hydrogen, substituted or unsubstituted lower alkyl or -COR where R_6 is selected from hydroxy, substituted or unsubstituted lower alkoxy, substituted or unsubstituted amino, glycine ester, or an activated leaving group; R_1 is selected from hydrogen, or substituted or unsubstituted lower alkyl; R_2 and R_3 are each selected from hydrogen or a thiol protecting group; and R_4, R_5, R_7, and R_8 are each selected from hydrogen or lower alkyl; and salts thereof. These complexes

were used primarily as renal function monitoring agents.

Arginylglycylaspartate (Arg-Gly-Asp *or* RGD) and derivative peptides are known to bind to blood clots (*see* U.S. Patent Nos. 4,792,525, 4,857,508 and 4,578,079) and RGD derivatives have been labeled with technetium as imaging agents, *Journal of Nuclear Medicine* 31, pp. 757, No. 209 (1990).

SUMMARY OF THE INVENTION

The invention encompasses polypeptides for labeling with technetium-99m and imaging target sites within a mammalian body comprising (a) a specific binding polypeptide region which specifically binds to the target site to be imaged, and (b) a technetium binding region of the formula Cp(aa)Cp wherein Cp is a protected cysteine and (aa) is an amino acid and wherein the technetium binding region is covalently bound to the specific binding polypeptide region. The invention includes technetium-99m complexes and methods for using the technetium-99m complexes to image target sites within a mammalian body.

DETAILED DESCRIPTION OF THE INVENTION

The Cp(aa)Cp technetium binding group is covalently linked to the specific binding polypeptide preferably by one or more amino acids, most preferably glycine. Alternatively, the Cp(aa)Cp technetium binding group may be directly covalently linked to the specific binding polypeptide or other covalent linking groups can be used such as bifunctional amino/carboxy compounds which are not naturally-occurring amino acids.

Representative specific binding polypeptide sequences are:

Atherosclerotic Plaque Binding Peptides

YRALVDTLK
RALVDTLK
RALVDTLKFVTQAEGAK
YAKFRETLEDTRDRMY
AKFRETLEDTRDRMY
YAALDLNAVANKIADFEL

2101942

Atherosclerotic Plaque Binding Peptides (cont'd.)

AALDLNAVANKIADFEL
YRALVDTLKFVTEQAKGA
RALVDTLKFVTEQAKGA
YRALVDTEFKVKQEAGAK
RALVDTEFKVKQEAGAK
YRALVDTLKFVTQAEGAK

Peptides Targeted to Infections and Atherosclerotic Plaque

VGVAPGVGVAPGVGVAPG
VPGVGVPGVGVPGVGVPGVG
formyl.Nleu.LF.Nleu.YK
formyl MIFL
formyl MLFK
formyl MLFI
formyl MFIL
formyl MFLI
formyl MLIF
formyl MILF
TKPR
VGVAPG
formyl MLF

Thrombus

NDGDFEEIPEEYLQ
NDGDFEEIPEEY(SO$_3$Na)LQ
GPRG

Platelets

D-Phe.PRPGGGGNGDFEEIPEEYL
RRRRRRRRRGDV
PLYKKIIKKLLES
RGD
RGDS

Infection and Atherosclerotic Plaque

YIGSR
CH_2CO.YIGSRC

Alzheimers Disease (Amyloid Plaque)

EKPLQNFTLSFR

[Single letter abbreviations for amino acids can be found in G. Zubay, Biochemistry (2d ed.), 1988, (MacMillan Publishing: New York), p. 33.]

In the Cp(aa)Cp, the Cp is a protected cysteine where the S-protecting groups are the same or different and may be but not limited to:

$-CH_2$-aryl (aryl is phenyl or alkyl or alkyloxy substituted phenyl);

$-CH-(aryl)_2$, (aryl is phenyl or alkyl or alkyloxy substituted phenyl);

$-C-(aryl)_3$, (aryl is phenyl or alkyl or alkyloxy substituted phenyl);

$-CH_2$-(4-methoxyphenyl);

-CH-(4-pyridyl)$(phenyl)_2$;

$-C(CH_3)_3$

-9-phenylfluorenyl;

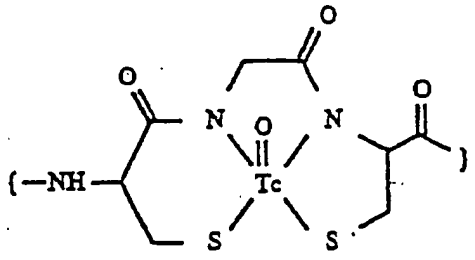
$-CH_2NHCOR$ (R is unsubstituted or substituted alkyl or aryl);

$-CH_2$-NHCOOR (R is unsubstituted or substituted alkyl or aryl);

-CONHR (R is unsubstituted or substituted alkyl or aryl);

$-CH_2$-S-CH_2-phenyl

When Cp-gly-Cp is combined with technetium, the following complex with the protecting groups removed is formed:



The preferred protecting group has the formula $-CH_2$-NHCOR wherein R is a lower alkyl having 1 and 8 carbon atoms, phenyl or phenyl-substituted with lower alkyl, hydroxyl, lower alkoxy, carboxy, or lower alkoxycarbonyl.

Compounds of the present invention can generally advantageously be

B

prepared on an amino acid synthesizer. Compounds of this invention are advantageous in that they are soluble and the sulfur is stabilized.

In forming the complex of radioactive technetium with the compounds of this invention, the technetium complex, a salt of technetium-99m pertechnetate, is reacted with the compound of this invention in the presence of a reducing agent such as stannous chloride ferrous ion or sodium dithionite. These technetium labeled complexes can also be made by exchange of a prereduced technetium -99m complex. The complexes are conveniently provided in a kit form comprising a sealed vial containing a predetermined quantity of a compound to be labeled and a sufficient amount of reducing agent to label the compound with technetium-99m. Alternatively, the complex may be formed by reacting the compound of this invention with a pre-formed labile complex of technetium and another compound. This process is known as ligand exchange, is well known to those skilled in the art, and the labile complex may be formed using such compounds as tartrate, citrate, gluconate or mannitol, for example. Among the technetium-99m pertechnetate salts are included the alkali metal salts such as the sodium salt or ammonium salts, or lower alkyl ammonium salts. The reaction of the compound of this invention with pertechnetate or preformed labile complex can be carried out in an aqueous medium at room temperature. The anionic complex which has a charge of -1 is formed in the aqueous medium in the form of a salt with a suitable cation such as sodium, ammonium cation, mono, di- or tri-lower alkyl amine cation, etc. Any conventional salt of the anionic complex with a pharmaceutically acceptable cation can be used in accordance with this invention.

In carrying out the reaction of the compounds of this invention with pertechnetate or a labile complex to form the anionic complex, the thiol protecting group is cleaved. Therefore, this reaction not only introduces the radioactive metal into the compound but also cleaves the thiol protecting group. All of the aforementioned thiol protecting groups are cleaved by a

reaction of salts of radioactive metals in accordance with this invention.

In forming the complex the radioactive material has a suitable amount of radioactivity. In forming the Tc-99m radioactive anionic complexes, it is generally preferred to form radioactive complexes in solutions containing radioactivity at concentrations of from about 0.01 milliCuries (mCi) to 100 mCi per ml.

The complex can be used for visualizing organs such as the kidney for diagnosing disorders in these organs, tumors and blood clots can also be imaged. In accordance with this invention, the anionic complex either as a complex or as a salt with a pharmaceutically acceptable cation is administered in a single unit injectable dose. Any of the common carriers such as sterile saline solution, plasma, etc., can be utilized after the radiolabeling for preparing the injectable solution to diagnostically image various organs, clots, tumors and the like in accordance with this invention. Generally, the unit dose to be administered has a radioactivity of about 0.01 mCi to about 100 mCi, preferably 1 mCi to 20 mCi. The solution to be injected at unit dosage is from about 0.01 ml to about 10 ml. After intravenous administration, imaging of the organ *in vivo* can take place in a matter of a few minutes. However, imaging can take place, if desired, in hours or even longer, after injecting into patients. In most instances, a sufficient amount of the administered dose will accumulate in the area to be imaged within about 0.1 of an hour to permit the taking of scintiphotos. Any conventional method of imaging for diagnostic purposes can be utilized in accordance with this invention.

The complexes may be administered intravenously in any conventional medium for intravenous injection such as an aqueous saline medium, or in blood plasma medium. Such medium may also contain conventional pharmaceutical adjunct materials such as, for example, pharmaceutically acceptable salts to adjust the osmotic pressure, buffers, preservatives and the like. Among the preferred mediums are normal saline and plasma.

The methods for making and labeling these compounds are more fully illustrated in the following examples.

Example 1

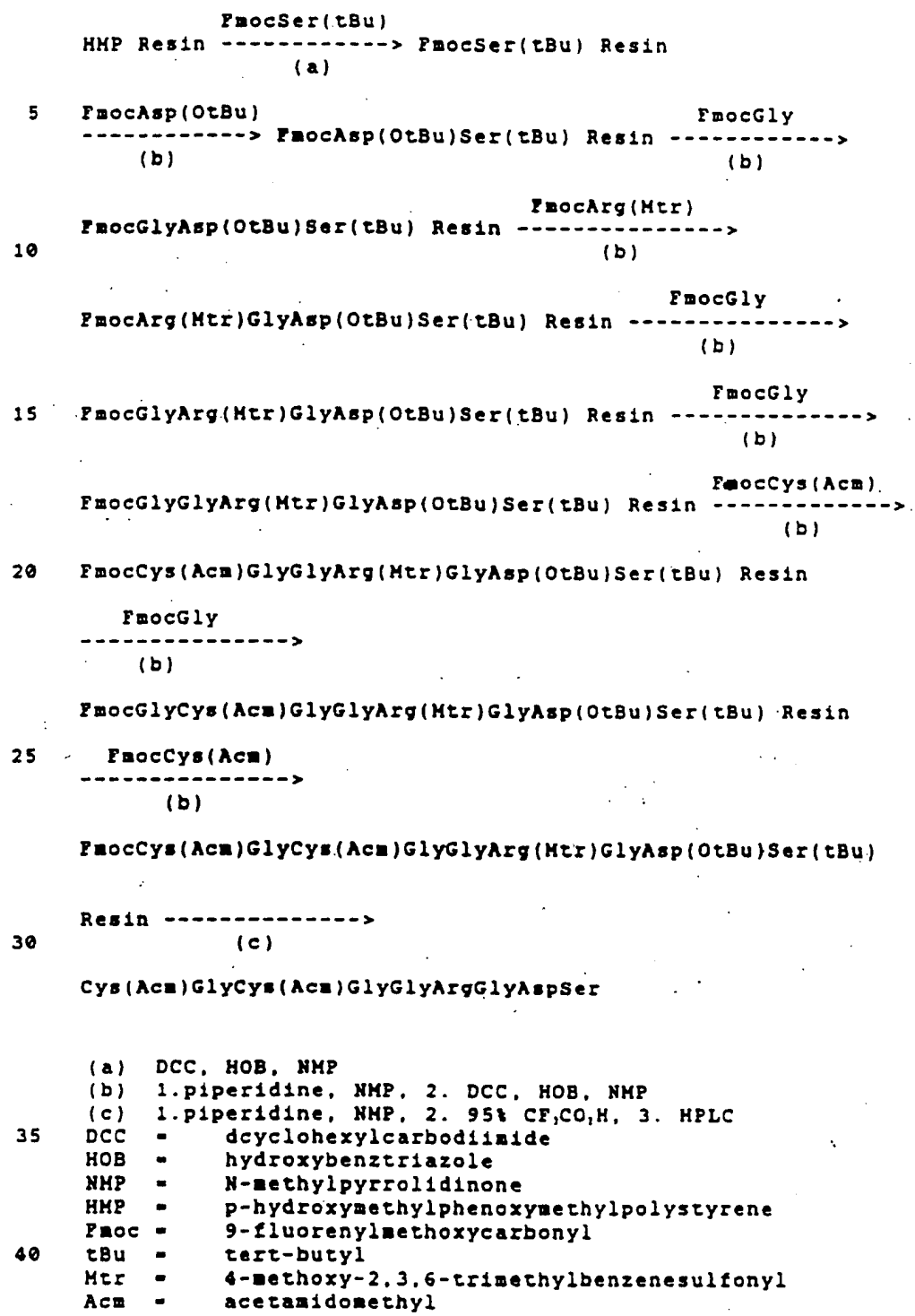
Cys(Acm)GlyCys(Acm)GlyGlyArgGlyAspSer

The title compound was prepared on a 0.25 millimole scale using an Applied Biosystems Model 431A peptide Synthesizer, N-terminus Fmoc protection and HMP resin (see Scheme). The product was cleaved from the resin using 95% trifluoroacetic acid at room temperature for 3 hours. Work-up and high performance liquid chromatography (HPLC) purification (using a Vydac*2.20cm x 25cm, 10um, C-18 column with a 20-minute gradient of 0.1% trifluoroacetic acid to 70% acetonitrile/ 0.1% trifluoroacetic acid at a flow rate of 25 ml/min) gave 50 mg of the title compound, 95% pure. (HPLC peak eluted at 5.5 min; Pos. ion FABMS Calc MW 952.97, Found 953).

* - Trade-mark

B

Scheme for Preparation of the Title Compound



(a) DCC, HOB, NMP
(b) 1.piperidine, NMP, 2. DCC, HOB, NMP
(c) 1.piperidine, NMP, 2. 95% CF_3CO_2H, 3. HPLC

DCC = dcyclohexylcarbodiimide
HOB = hydroxybenztriazole
NMP = N-methylpyrrolidinone
HMP = p-hydroxymethylphenoxymethylpolystyrene
Fmoc = 9-fluorenylmethoxycarbonyl
tBu = tert-butyl
Mtr = 4-methoxy-2,3,6-trimethylbenzenesulfonyl
Acm = acetamidomethyl

Example 2

Radiolabeling of Compound of Example 1 with Tc-99m

0.3 mg of the compound prepared as in Example 1 was dissolved in 0.3 ml of 0.05M potassium phosphate buffer (pH 7.4) containing 0.5 mM EDTA. Tc-99m gluceptate was prepared by reconstituting a Glucoscan* vial (E.I. DuPont de Nemours, Inc.) with 1.0 ml of Tc-99m sodium pertechnetate containing 26 mCi. After 15 minutes at room temperature, 75 ul of Tc-99m gluceptate was added to 0.3 mg of the compound prepared as in Example 1 and boiled for 45 minutes.

The extent of Tc-99m labeling of the peptide was determined by chomatography using Merck silica gel 60 F_{250} aluminum-backed strips which were spotted with 10 ul of sample and chromatographed with acetonitrile:0.5M sodium chloride solvent (15:85) approximately 2% of Tc-99m radioactivity remained at R_f 0.0, confirming that no significant Tc-99m colloids or aggregates were generated.

The Tc-99m labeled peptide purity was determined by HPLC using a Brownlee Spheri-5*(5um) resin, RP-18, 220 x 4.6 mm column and the following gradient: 0% A (CH_3CN:H_2O:TFA, 70:30:0.1) and 100% B (0.1% TFA in H_2O) to 100% A + 0% B over 10 minutes at 1.5 ml/min; and then held at the 100% A solvent for 5 minutes. This protocol yielded 100% of the radiometric species detected (by in-line NaI detector) as a single species (retention time = 10.9 min). Tc-99m gluceptate and Tc-99m sodium pertechnetate elute between 1 and 4 minutes under identical conditions, confirming the identity of the Tc-99m labeled peptide isolated.

* - Trade-mark

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A reagent for labeling with technetium-99m and imaging target sites within a mammalian body comprising:

   (a) a specific binding peptide comprising 3 to 100 amino acids which specifically binds to the target site; and

   (b) a technetium binding region covalently bound to the peptide region and having a formula:

   Cp(aa)Cp

   wherein Cp is a cysteine having a protected thiol group and (aa) is an amino acid.

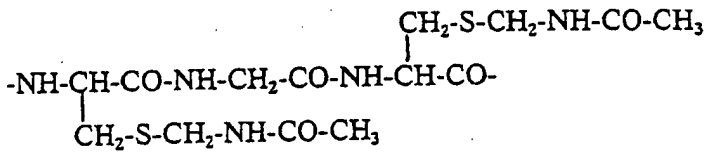
2. A reagent according to claim 1, wherein the specific binding peptide and Cp(aa)Cp is covalently linked through from about one to twenty amino acids.

3. A reagent according to claim 1, wherein the protected cysteine has a protecting group of the formula:

   $-CH_2-NH-CO-R$

   wherein R is a lower alkyl having 1 to 8 carbon atoms, phenyl, or phenyl substituted with lower alkyl, hydroxy, lower alkoxy, carboxy, or lower alkoxycarbonyl, or 2-,3-,4-pyridyl.

4. A reagent according to claim 1, wherein Cp(aa)Cp has the formula:



5. A reagent according to claim 1, wherein the specific binding peptide region specifically binds to clots, tumors, sites of infection, atherosclerotic plaques, amyloid plaques or bone.

B

6. A reagent according to claim 1, wherein the specific binding peptide region is selected from polypeptides consisting of the amino acid sequences:

YRALVDTLK

RALVDTLK

RALVDTLKFVTQAEGAK

YAKFRETLEDTRDRMY

AKFRETLEDTRDRMY

YAALDLNAVANKIADFEL

AALDLNAVANKIADFEL

YRALVDTLKFVTEQAKGA

RALVDTLKFVTEQAKGA

YRALVDTEFKVKQEAGAK

RALVDTEFKVKQEAGAK

YRALVDTLKFVTQAEGAK

VGVAPGVGVAPGVGVAPG

VPGVGVPGVGVPGVGVPGVG

formyl.Nleu.LF.Nleu.YK

formyl MIFL

formyl MLFK

formyl MLFI

formyl MFIL

formyl MFLI

formyl MLIF

formyl MILF

TKPR

VGVAPG

formyl MLF

NDGDFEEIPFFYLQ

NDGDFEEIPEEY(SO_3Na)LQ

B'

GPRG

D-Phe.PRPGGGGNGDFEEIPEEYL

RRRRRRRRRGDV

PLYKKIIKKLLLES

RGD

RGDS

YIGSR

CH_2CO.YIGSRC

EKPLQNFTLSFR

7. The reagent of claim 6, bound to technetium-99m.

8. A complex formed by reacting a reagent of claim 1, with technetium-99m in the presence of a reducing agent.

9. The complex of claim 8, wherein the said reducing agent is selected from the group of a dithionite ion, a stannous ion, or a ferrous ion.

10. A complex formed by labelling a reagent of claim 1, with technetium-99m by ligand exchange of a prereduced technetium-99m complex.

11. A kit for preparing a radiopharmaceutical preparation, said kit comprising sealed vial containing a predetermined quantity of a reagent of claim 1, and a sufficient amount of reducing agent to label said compound with technetium-99m.

12. A method for imaging a target site within a mammalian body comprising administering an effective diagnostic amount of a polypeptide of claim 1, which is labeled with technetium-99m, and wherein the specific binding peptide region binds to the target site, and detecting the

localized technetium-99m.

13. A process of preparing the peptide according to claim 1, wherein the peptide is chemically synthesis *in vitro*.

14. The process of preparing the peptide according to claim 13, wherein the peptide is synthesized by solid phase peptide synthesis.